

**What is claimed is:**

1. A method for amplifying an unknown nucleotide sequence adjacent to a known nucleotide sequence, which comprises the step of (a) performing a primary amplification of said unknown nucleotide sequence using a DNA walking annealing control primer (DW-ACP) and a first target-specific primer; in which said step (a) comprises:
  - (a-1) performing a first-stage amplification of said unknown nucleotide sequence at a first annealing temperature, comprising at least one cycle of primer annealing, primer extending and denaturing using a first degenerate DW-ACP containing a degenerate random nucleotide sequence to hybridize with said unknown nucleotide sequence and a hybridizing nucleotide sequence substantially complementary to a site on said unknown nucleotide sequence, wherein said first annealing temperature enables said first degenerate DW-ACP to function as a primer, whereby a first degenerate DW-ACP extension product is generated; and
  - (a-2) performing a second-stage amplification at a second annealing temperature to render said first degenerate DW-ACP not to function as a primer, comprising:
    - (a-2-1) amplifying said first degenerate DW-ACP extension product using said first target-specific primer to hybridize with a target-specific nucleotide sequence substantially complementary to a site on said known nucleotide sequence, whereby a target-specific primer extension product is generated,
    - (a-2-2) amplifying said target-specific primer extension product using a second DW-ACP to hybridize with a nucleotide sequence complementary to said first degenerate DW-ACP sequence of said target-specific primer extension product, whereby a second DW-ACP extension product is generated, and
    - (a-2-3) amplifying said second DW-ACP extension product using said second DW-ACP and said first target-specific primer, whereby a primary amplification product without a degenerate random nucleotide sequence is generated.

2. The method according to claim 1, wherein said first-stage amplification is performed for one cycle.
3. The method according to claim 1, wherein said second-stage amplification is performed for 5 at least 5 cycles.
4. The method according to claim 1, wherein said first annealing temperature is between about 35°C and 50°C.
- 10 5. The method according to claim 1, wherein said second annealing temperature is between about 50°C and 72°C.
6. The method according to claim 1, wherein said first degenerate DW-ACP has a general formula I:  
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$$5'-X_p-Y_q-Z_r-Q_s-3' \quad (\text{I})$$

wherein,  $X_p$  represents a 5'-end portion having a pre-selected nucleotide sequence,  $Y_q$  represents a regulator portion comprising at least two universal base or non-discriminatory base analog residues,  $Z_r$  represents a degenerate random sequence portion having a degenerated random nucleotide sequence,  $Q_s$  represents a 3'-end portion having a hybridizing nucleotide sequence substantially complementary to a site on said unknown nucleotide sequence to hybridize therewith,  $p$ ,  $q$ ,  $r$  and  $s$  represent the number of nucleotides, and  $X$ ,  $Y$ ,  $Z$  and  $Q$  are deoxyribonucleotide or ribonucleotide.
- 20 7. The method according to claim 6, wherein said regulator portion in said first degenerate DW-ACP is capable of restricting the annealing portion of said primer to its 3'-end portion at said first annealing temperature.
- 25 8. The method according to claim 6, wherein said second DW-ACP has a general formula II:  
$$5'-X'_p-S_u-Y'_v-Z'_w-3' \quad (\text{II})$$

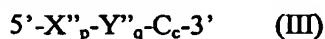
wherein,  $X'_p$  represents a 5'-end portion having a nucleotide sequence corresponding to the 5'-end portion of the first degenerate DW-ACP,  $S_u$  represents a supplementary annealing portion comprising a nucleotide sequence to hybridize with a portion opposite to the regulator portion of the first degenerate DW-ACP in the target-specific primer extension product of the 5 step (a-2-1),  $Y'_v$  represents a regulator portion comprising at least two universal base or non-discriminatory base analog residues and prevents annealing of said  $X'_p$  and  $S_u$  portions to non-target sequences except to the nucleotide sequence complementary to the first degenerate DW-ACP,  $Z'_w$  represents a 3'-end portion having a nucleotide sequence corresponding to the 3'-end portion of the first degenerate DW-ACP, p, u, v and w represent the number of nucleotides, and 10  $X'$ ,  $S'$ ,  $Y'$ , and  $Z'$  are deoxyribonucleotide or ribonucleotide.

9. The method according to claim 1, wherein said method further comprises the step of (c) performing a secondary amplification at a third annealing temperature, comprising at least one cycle of primer annealing, primer extending and denaturing, using a third DW-ACP comprising 15 at its 3'-end portion a nucleotide sequence to hybridize with the opposite-sense nucleotide sequence to said second DW-ACP sequence present at the 3'-end of said primary amplification product and said first target-specific primer of the step (a) or a nested target-specific primer designed to amplify an internal region of said primary amplification product.

20 10. The method according to claim 9, wherein said third annealing temperature ranges from about 50°C and 72°C.

11. The method according to claim 9, wherein said method further comprises the step (b) of purifying a reaction resultant of the step (a) to remove said first degenerate DW-ACP, said 25 second DW-ACP and said first target-specific primer prior to performing the step (c).

12. The method according to claim 9, wherein said third DW-ACP has a general formula III:



wherein,  $X''_p$  represents a 5'-end portion having a nucleotide sequence corresponding to

all or a part of the 5'-end portion of said second DW-ACP, Y"<sub>q</sub> represents a regulator portion comprising at least two universal base or non-discriminatory base analog residues corresponding to the supplementary annealing portion, a part of the 5'-end portion, a part of supplementary annealing portion plus a part of the 5'-end portion, or a part of supplementary annealing portion plus a part of the regulator portion of the second DW-ACP of the formula II and prevents annealing of said X"<sub>p</sub> portion to non-target sequences of said primary amplification product except to the nucleotide sequence complementary to said second DW-ACP, C<sub>c</sub> represents a 3'-end portion having a nucleotide sequence to hybridize with the opposite-sense nucleotide sequence to all or a part of the 3'-end portion and regulator portion sequences of said second DW-ACP, p, q and c represent the number of nucleotides, and X", Y", and C are deoxyribonucleotide or ribonucleotide.

13. The method according to claim 1, wherein said nucleotide sequence to be amplified is gDNA or cDNA.

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14. The method according to any one of claims 6, 8 and 12, wherein said universal base or non-discriminatory base analog residue is selected from the group consisting of deoxyinosine, inosine, 7-deaza-2'-deoxyinosine, 2-aza-2'-deoxyinosine, 2'-OMe inosine, 2'-F inosine, deoxy 3-nitropyrrole, 3-nitropyrrole, 2'-OMe 3-nitropyrrole, 2'-F 3-nitropyrrole, 1-(2'-deoxy-beta-D-ribofuranosyl)-3-nitropyrrole, deoxy 5-nitroindole, 5-nitroindole, 2'-OMe 5-nitroindole, 2'-F 5-nitroindole, deoxy 4-nitrobenzimidazole, 4-nitrobenzimidazole, deoxy 4-aminobenzimidazole, 4-aminobenzimidazole, deoxy nebularine, 2'-F nebularine, 2'-F 4-nitrobenzimidazole, PNA-5-introindole, PNA-nebularine, PNA-inosine, PNA-4-nitrobenzimidazole, PNA-3-nitropyrrole, morpholino-5-nitroindole, morpholino-nebularine, morpholino-inosine, morpholino-4-nitrobenzimidazole, morpholino-3-nitropyrrole, phosphoramidate-5-nitroindole, phosphoramidate-nebularine, phosphoramidate-inosine, phosphoramidate-4-nitrobenzimidazole, phosphoramidate-3-nitropyrrole, 2'-0-methoxyethyl inosine, 2'0-methoxyethyl nebularine, 2'-0-methoxyethyl 5-nitroindole, 2'-0-methoxyethyl 4-nitrobenzimidazole, 2'-0-methoxyethyl 3-nitropyrrole, and combinations thereof.

15. The method according to 14, wherein said universal base or non-discriminatory base analog residue is deoxyinosine, inosine, 1-(2'-deoxy-beta-D-ribofuranosyl)-3-nitropyrrole or 5-nitroindole.

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16. The method according to 15, wherein said universal base or non-discriminatory base analog residue is deoxyinosine.

17. The method according to any one of claims 6, 8 and 12, wherein said regulator portion  
10 comprise contiguous nucleotides having universal base or non-discriminatory base analog residue.

18. The method according to any one of claims 6, 8 and 12, wherein p represents an integer of  
10 to 60.

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19. The method according to any one of claims 6, 8 and 12, wherein q or u is at least 3.

20. The method according to any one of claims 6, 8 and 12, wherein q or u represents an  
integer of 2 to 10.

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21. The method according to claim 6 or 8, wherein r or v represents an integer of 2 to 5.

22. The method according to claim 6 or 8, wherein s or w represents an integer of 3 to 10.

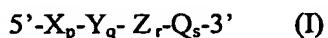
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23. The method according to claim 12, wherein c represents an integer of 5 to 15.

24. The method according to claim 8, wherein S comprises at least 2 contiguous  
deoxyguanosine nucleotides.

25. The method according to claim 12, wherein C comprises at least 2 contiguous deoxyguanosine nucleotides at a site to hybridize with the opposite-sense nucleotide sequence to the regulator portion sequences of said second DW-ACP.

5 26. A DNA walking annealing control primer for amplifying an unknown nucleotide sequence adjacent to a known nucleotide sequence, which is represented by the following general formula I:



wherein,  $X_p$  represents a 5'-end portion having a pre-selected nucleotide sequence,  $Y_q$  represents a regulator portion comprising at least two universal base or non-discriminatory base analog residues,  $Z_r$  represents a degenerate random sequence portion having a degenerated random nucleotide sequence,  $Q_s$  represents a 3'-end portion having a hybridizing nucleotide sequence substantially complementary to a site on said unknown nucleotide sequence to hybridize therewith,  $p$ ,  $q$ ,  $r$  and  $s$  represent the number of nucleotides, and  $X$ ,  $Y$ ,  $Z$  and  $Q$  are 10 deoxyribonucleotide or ribonucleotide.

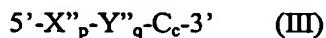
15 27. A DNA walking annealing control primer for amplifying an unknown nucleotide sequence adjacent to a known nucleotide sequence, which is represented by the following general formula II:



20 wherein,  $X'_p$  represents a 5'-end portion having a nucleotide sequence corresponding to the 5'-end portion of the first degenerate DW-ACP,  $S_u$  represents a supplementary annealing portion comprising a nucleotide sequence to hybridize with a portion opposite to the regulator portion of the first degenerate DW-ACP in the target-specific primer extension product of the step (a-2-1),  $Y'_v$  represents a regulator portion comprising at least two universal base or non-discriminatory base analog residues and prevents annealing of said  $X'_p$  and  $S_u$  portions to non-target sequences of the amplified product of the step (a) except to the nucleotide sequence complementary to the first degenerate DW-ACP,  $Z'_w$  represents a 3'-end portion having a nucleotide sequence corresponding to the 3'-end portion of the first degenerate DW-ACP,  $p$ ,  $u$ ,

v and w represent the number of nucleotides, and X', S, Y', and Z' are deoxyribonucleotide or ribonucleotide.

28. A DNA walking annealing control primer for amplifying an unknown nucleotide  
5 sequence adjacent to a known nucleotide sequence, which is represented by the following  
general formula III:



wherein,  $X''_p$  represents a 5'-end portion having a nucleotide sequence corresponding to all or a part of the 5'-end portion of said second DW-ACP,  $Y''_q$  represents a regulator portion comprising at least two universal base or non-discriminatory base analog residues corresponding to the supplementary annealing portion, a part of the 5'-end portion, a part of supplementary annealing portion plus a part of the 5'-end portion, or a part of supplementary annealing portion plus a part of the regulator portion of the second DW-ACP of the formula II and prevents annealing of said  $X''_p$  portion to non-target sequences of said primary 15 amplification product except to the nucleotide sequence complementary to said second DW-ACP,  $C_c$  represents a 3'-end portion having a nucleotide sequence to hybridize with the opposite-sense nucleotide sequence to all or a part of the 3'-end portion and regulator portion sequences of said second DW-ACP, p, q and c represent the number of nucleotides, and X'', Y'', and C are deoxyribonucleotide or ribonucleotide.

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29. A kit for amplifying an unknown nucleotide sequence adjacent to a known nucleotide sequence, which comprises the DNA walking annealing control primer of claim 26, the DNA walking annealing control primer of claim 27, the DNA walking annealing control primer of claim 28 or combinations thereof.

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30. Use of the method of claim 1 for a process involving nucleic acid amplification of unknown nucleotide sequence adjacent to a known nucleotide sequence.